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Implications of Candidatus Phytoplasma mali infection on phloem function of apple trees

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Apple proliferation (AP) is caused by a psyllid-transmitted phytoplasma and is one of the most economically important diseases on apple in Europe. AP was first reported in northern Italy and in the following years from many European countries. Previous research on phytoplasma-induced diseases of rosaceous fruit trees (mainly AP, pear decline and European stone fruit yellows), exhibits strong evidence that phloem injuries play a central and probably universal role in phytoplasma pathogenicity. The effect of phytoplasma infections on phloem function and the resulting diseases received little attention in the past. Phytoplasma infection severely impairs assimilate translocation in host plants and might be responsible for massive changes in phloem physiology including signalling components. As shown for other phytoplasma species, infection brings about Ca2+ influx into sieve tubes, leading to sieve-tube occlusion by callose deposition or protein plugging, which is assumed also for AP phytoplasma. Effectors may cause gating of sieve-element Ca2+ channels leading to sieve-tube occlusion with presumptive dramatic effects on phytoplasma spread, photosynthesis and distribution and the whole phloem physiology. However, there is indication that phloem loading is affected by phytoplasma infection. As sieve elements need a permanent input of energy to ensure their viability, sugar metabolism and the associate energy production of the companion cells have a dramatic impact on the physiological fitness of phloem function. It is presumptive that signalling substances are produced prior sieve-element occlusion to ensure the spread throughout the plant body. Analyses of diverse phytohormones in response to challenge with AP phytoplasma show a strong increase of salicylic acid accompanied by a decrease of jasmonic acid. This supports the idea that phytohormones have a role in plant defence signalling against phytoplasma infection. It is a matter of debate whether mechanisms involved in phloem impairment could differ between pathosystems and vary with the plant susceptibility to infection.

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Introduction

Economical significance of phytoplasma-induced diseases

Phytoplasmas cause diseases in more than 1000 plant species worldwide (Seemüller et al. 2002). In Europe, mainly perennial plants such as fruit trees and shrubs, grapevine, a range of forest and landscape trees, and some ornamentals are known to be affected. Among the fruit crops grown in Europe, apple proliferation (AP), apricot chlorotic leaf roll, leptonecrosis of Prunus salicina plums, pear decline as well as phytoplasma diseases of grapevine are of particular importance by impairing size and quality of the crop and vigor and longevity of the trees. The economic losses of phytoplasma diseases are difficult to assess. For apple proliferation a loss of 125 mio € i.e. has been estimated for Germany and Italy due to production of tasteless fruits (Strauss 2009). Additional expenses accrue for plant protection measures like insecticide applications or roguing. The diseases of pome- and stonefruits are caused by the closely related species Candidatus Phytoplasma mali, Ca. P. prunorum and Ca. P. pyri, respectively, that form a distinct group within the phylogenetic phytoplasma clade (Seemüller and Schneider 2004), while the grapevine diseases are caused by other more distantly related phytoplasmas. Due to their economic importance, the temperate fruit tree pathogens are among the most extensively studied phytoplasmas.
**Characteristics of phytoplasmas**

Phytoplasmas are plant-pathogenic prokaryotes of the class Mollicutes (mycoplasmas) characterized by the lack of a rigid cell wall and a small (0.53 - 1.35 Mb), low-GC (21 – 28%) genome (Kube et al. 2008; Marcone et al. 1999). They are most closely related to other wall-less bacteria such as the saprophytic acholeplasmas and, more distantly, to the human- and animal-pathogenic and commensal mycoplasmas. In plants, phytoplasmas inhabit almost exclusively the phloem sieve elements and are transmitted within their plant hosts by phloem sap-feeding homopterous insects, primarily leafhoppers (Cicadellidae) and, less frequently, planthoppers (Fulgoroidea) and psyllids (Psylloidea, Figure 1) (Seemüller et al. 2002).

**Phytoplasma life cycle.** Red dots represent the phytoplasmas and red arrows show the spread direction.

Phytoplasma infections are systemic, persistent and difficult to control. Due to the inability to cultivate phytoplasmas under axenic conditions, many biological aspects including their phytopathogenic traits are poorly understood. Thus far, the most important information on pathogenic and metabolic traits of these pathogens was obtained from complete genome sequences of six phytoplasmas that include Ca. P. mali (Kube et al. 2008). The remaining five are representatives of the aster yellows (Ca. P. asteris)/stolbur (Ca. P. solani) phytoplasma complex. Facilitated by genomic sequence data, four effector proteins related to virulence were identified in genomes of two Ca. P. asteris strains that induce witches’ broom (WB) formation and dwarfism (Hoshi et al. 2009) or target the nuclei of the plant and may alter plant cell physiology (Sugio et al. 2011). None of these effectors or other virulence-related factors were detected in phytoplasmas infecting rosaceous fruit trees, that are only distantly related to Ca. P. asteris or Ca. P. solani. However, phytoplasma induced phloem injuries were observed in diseased fruit trees leading to severe symptoms and therefore may play a central and probably universal role in phytoplasma pathogenicity.

**Life cycle**

Phytoplasmas are bacteria that live in and manipulate plants and insects (Hogenhout et al. 2008). They require replication in both hosts for their survival. Phytoplasmas are ingested with the plant/phloem sap and move through the insect stylet and invade gut, epithelial and muscle tissues, hemolymph and salivary glands. They multiply in salivary gland cells and are transported with the saliva back into the phloem tissue of new host plants (Figure 2). There is evidence that some phytoplasmas are vertically transmitted to their progeny (Hanboonsong et al. 2002), or transmitted via seeds as shown for coconut embryos (Cordova et al. 2003), however, the predominant route means of survival of phytoplasmas is through transmission between insects and plants. They appear to manipulate their insect and plant hosts to enhance their own transmission efficiencies. In plants, they remain mainly restricted to the phloem tissue (Doi et al. 1967; Whitcomb and Tully 1989), and spread throughout the plant by moving through the pores of the sieve plates that divide the phloem sieve tubes (Figure 2). As mature sieve tubes usually contain the highest concentration of phytoplasmas (Christensen et al. 2004), they were also found inside the cytoplasm of immature phloem elements. Next to sieve tubes, phytoplasmas have been detected in the cytoplasm of phloem parenchyma cells adjacent to sieve elements (Sears and Klomparens 1989; Siller et al. 1987).
plants could be infected but some reacted hypersensitive whereas others were as tolerant as commercial rootstocks. Symptom development also depends on the virulence of the infecting AP phytoplasma strain as shown in a study of 24 Ca. P. mali accessions of different origin (Seemüller and Schneider 2007). Virulent (Figure 3B), moderately virulent and avirulent (Figure 3A and D) accessions were present in almost equal numbers. The plant reaction due to the virulence of the pathogen is also evident in the experimental phytoplasma host Catharanthus roseus as exemplified with the avirulent and virulent apple proliferation strain 1/93 and 12/93, respectively (Figure 3D-E).

The plant habitat of AP phytoplasmas
The plant vascular system serves as main route for the long- and short-distance transport of various compounds throughout the plant (van Bel 1996; Hafke et al. 2005). It consists of two major tissue types—xylem, which conducts water and nutrients, and phloem, which transports mainly organic compounds.

Sieve elements (SEs), companion cells (CCs) and phloem parenchyma cells (PPCs) are the three phloem elements involved in long-distance transport of photoassimilates (van Bel 1996; Hafke et al. 2005) and several long-distance signals, such as diverse RNA-species, proteins, nitric oxide, azelaic acid, SFD1/GLY1-derived glycerol-3-phosphate, dehydroabietinal, phytohormones (e.g. salicylic acid, jasmonates), as well as electrical signals (e.g. Davies 2006; Park et al. 2007; Gaupels et al. 2008; Jung et al. 2009; Dempsey and Klessig 2012; Furch et al. 2014). While the enucleate SEs form the transport pathway, the CCs are engaged in maintaining their viability. A high density of pore-plasmodesma units (PPUs) and tight endoplasmic reticulum (ER) coupling SE and CC underlines an intimate symplasmic connection across this boundary (Kempers et al. 1998; Martens et al. 2006). Permanent metabolic and energetic support for the SEs is required since their cellular machinery is largely reduced during ontogeny to make way for a more efficient mass flow (van Bel and Knoblauch 2000).

As phytoplasmas are obligate parasites, living in the phloem system and spread throughout the plant by moving through the pores of the sieve plates (Figure 4), an impairment of the phloem is very likely.

Phytoplasma-induced injuries of the phloem were first identified by Schneider (1973). He found that severe phytoplasma infections of pear are associated with the pathological deposition of callose inside the sieve elements followed by the collapse of these SEs and their CCs. This loss of conducting tissue may result in a hyperactivity of the cambium, leading to the formation of a more or less pronounced replacement phloem. As this tissue usually becomes similarly affected, starch accumulates in the stem, especially in the leaves, whereas the roots are starving. Depending on the susceptibility of the genotype, diseased trees decline or suffer more or less (Schneider 1976; Schaper and Seemüller 1982; Kartte and Seemüller 1991). The phloem system of all higher plants pervades the plant from roots to leaf tips and ensures communication and nutrient supply among all plant organs and tissues. A potential loss of vascular sap due to a wound is prevented via efficient sealing mechanisms with callose and proteins (Knoblauch et al. 2003; Furch et al. 2014).
Thus, phloem sap cannot be usually collected by wounding of the vascular system, as known for cucurbits and Ricinus (Dinant and Lemoine 2010; Zimmermann et al. 2013). Interestingly, considerable volumes of phloem exudate can be collected from severely affected trees (Figure 5A) but not from infected non-symptomatic or healthy trees. The great volume of ~300 µl per droplet, the numerous proteins and the pH of 7.5-8 indicates sieve-tube content (Figure 5). High protein concentrations between 0.07 and 110 mg ml⁻¹ with 150-1000 different proteins were found in sieve-tube exudates of various plant species (e.g. Schobert et al. 1998; Rodriguez-Medina 2009; Zimmermann et al. 2013).

The pH value of the cytosol, respectively sieve-tube exudate, is a characteristic, regulated and consistent trait (Oja et al. 1999; Felle 2001, 2005; Hafke et al. 2005) and is probably not or equally influenced by the influx of xylem water after wounding (Zimmermann et al. 2013). These findings indicate that the phytoplasma infection impairs the sealing mechanism of the phloem (Kollar et al. 1989). As has been shown for several other bacterial plant pathogens, it is likely that phytoplasmas produce a series of virulence proteins that suppress plant responses (Hogenhout et al. 2008).

Some phytoplasmas induce severe phloem necrosis in their host plants, indicating that these plants react to the phytoplasma infection. However, many phytoplasmas, do not seem to induce phloem necrosis but still accumulate to high densities in phloem elements.

Phloem located plant defence

It is a permanent challenge for plants to protect themselves against the attack and harmful effects of pathogens. In particular, the phloem is one preferred destination for plant pathogens, as it mediates the translocation of primary and secondary metabolites. Hence, the phloem reflects an interesting propagation pathway and an important source of energy and defence-related information. Compromised phloem functionality may have disastrous consequences for plant health and development. Focus on phloem research over the past years has been shifted from the translocation of nutrients towards signalling and defence. One example is the occlusion of SEs.

Sieve-element occlusion

The SE sealing prevents an invasion and manifestation of pathogens and their released effectors/elicitors, redirects nutrient flows, and enriches signal and defence molecules and may be seen as a general defence response to pathogen attack. Sieve tubes may be constricted by callose plugged by proteins (e.g. Furch et al. 2007). Callose deposition is an universal mode of sieve-plate occlusion (Kudlicka and Brown 1997; Nakashima et al. 2003). It is a 6-1.3-glucan polymer that is produced enzymatically and deposited extracellularly around plasmodesmata and sieve pores in the form of collars (Blackman et al. 1998; Zabotin et al. 2002) as a reaction to chemical or mechanical stress (Kudlicka and Brown 1997; Nakashima et al. 2003; Levy et al. 2007). As for protein plugging, the numerous aggregation forms of phloem-specific proteins (Cronshaw and Sabnis 1990) display an immense variation within the plant kingdom. Spindle-like protein bodies that only occur in Fabaceae (Palevitz and Newcomb 1971) were observed in Vicia faba sieve tubes. Triggered by osmotic shock or Ca²⁺ application, these protein bodies designated as forisomes (Knoblauch et al. 2003) disperse directly and recondense “spontaneously” into the original state in intact sieve tubes (Knoblauch et al. 2001, 2003). Manipulation of isolated forisomes showed a strong calcium-dependent dispersion/recondensation.

Transient influx of Ca²⁺ constitutes an early element of signalling cascades triggering pathogen defence responses
in plant cells. Numerous studies have provided evidence that Ca²⁺ plays a pivotal role in activating the plant’s surveillance system against attempted microbial invasion (Yang et al. 1997; Scheel 1998). Stimulus specific and spatio-temporal defined Ca²⁺ signatures of characteristic magnitude, frequency, and duration are assumed to signal specificity of transduction cascades (Thulau et al. 1998; Trewavas 1999). Subsequently, the binding of cytosolic Ca²⁺ to, for example, calmodulin, Ca²⁺-dependent protein kinases, phosphatases and Ca²⁺-gated ion channels facilitates downstream signal transduction directed toward activation of a signal-specific cellular response (Blumwald et al. 1998).

V. faba sieve tubes infected by a Stolbur phytoplasma contain consistently dispersed forisomes hinting at Ca²⁺ levels appreciably higher than in healthy sieve tubes (Musetti et al. 2013). Moreover, the sieve tubes are sealed with thick deposits of callose (Musetti et al. 2013). Undoubtedly, phytoplasmas induce Ca²⁺ influx with inherent consequences for forisome dispersion and callose synthesis. As V. faba and Stolbur phytoplasma is an exclusive artificial system, it would be of interest to study how a pathogen interacts with a natural host (for example Malus and AP phytoplasma).

The above reported effect of the impairment of the sealing mechanism is unexpected because both occlusion mechanisms and the induced phloem necrosis reduce or inhibit the mass flow. The findings indicate conditions, depending on the phytoplasma titer or the strain (avirulent or virulent) where the sieve tubes are still functioning but where the sealing mechanism is affected. So far, it has been unclear if SE occlusion (may be reversible) is part of the plant’s strategy against phytoplasma spread or if phytoplasmas induce and explore symplasmic isolation for an undispatch multiplication (van Bel et al. 2014). Hence, one major future task should be to understand the physiological relationship of phytoplasma infection, sieve-tube occlusion and wound induced vascular exudation.

Stress-related changes of phytohormones and proteins

The formation of WB, the small-fruit symptom, and the reduced root growth of diseased apple trees can be explained with an infection-induced hormonal imbalance, in particular by an altered auxin (Indole-3-acetic acid; IAA)/cytokinin ratio (Hegele and Bangerth 1997). There is indication that synthesis, metabolism and/or transport of plant hormones are affected in diseased plants (Hare 1977). Phytohormone dispersion in leaves of Ca. P. mali-infected (virulent and avirulent) and healthy apple trees (Figure 6), show significant phytoplasma-triggered effects for all examined phytohormones (Figure 6A: IAA-auxin, ABA-abscisic acid, SA-salicylic acid; Figure 6B: JA-jasmonic acid, cis-OPDA-cis-12-oxophytodienoic acid,JA-Ile-(+) -7-iso-jasmonoyl-1-leucine). This is consistent with a role in immune signalling (Dempsey and Klessig 2012; Pieterse et al. 2012). It is generally accepted that pathogens with a biotrophic lifestyle are more sensitive to SA-mediated induced defence mechanisms (Glazebrook 2005), hence a significant increase of SA is expected. The increase of SA in plants infected by the virulent and avirulent strain by either 100% and a parallel decrease of JA and JA-Ile points to a cross-talk and indicate that an innate immunity response has been initiated (Kornneef and Pieterse 2008). The downregulation of the JA-mediated defences may help the plant to minimize energy costs and allows the plant to fine-tune its defence response (Pieterse et al. 2001; Bostock 2005; Kornneef and Pieterse 2008). ABA and IAA have also been implicated in plant defence, but their significance is less well studied (Kornneef and Pieterse 2008). ABA is produced in response to microbes (De Vleeschauwer et al. 2010), transported from the producing root cells into the xylem and released to the above-ground plant parts (Zang et al. 2008; Furfert et al. 2014). IAA has profound effects on plant growth and development. Both plants and some plant pathogens can produce IAA to modulate plant growth (Zhao 2010). It is very likely that the significantly increased IAA level is responsible for formation of WB. At present it is not known, if the plants produce more IAA due to the phytoplasma infection, or if the phytoplasmas itself produce IAA.

**Figure 6:** Analysis of phytohormones in leaves of healthy apple trees and trees infected by either an avirulent (a-vir) or a virulent (vir) Ca. P. mali strain. Each bar represents the mean of 13-25 replicates from 6 different plants ± standard deviation. The statistical significance is tested with one-way ANOVA by pairwise comparison with Students t-test and marked with different letters (p = 0.05). 250 mg of either leaf material was ground and extracted in the presence of a mix of internal phytohormone standards (Vadassery et al. 2012). Subsequently, the reduced and resolved sample was loaded onto a reversed-phase column (Oasis HLB 3 cc; Waters, Eschborn, Germany) that was first pre-conditioned by the addition of 2 ml methanol (MeOH) followed by 2 ml water. After being washed with 2 ml 5% (v/v) MeOH/water solution containing 0.5% (v/v) formic acid, the column was eluted with 2 ml MeOH. The eluate was completely dried in a speed-vac, resolved in 0.1 ml MeOH and subsequently subjected to liquid chromatography–mass spectrometry analysis according to Vadassery et al. (2012).
membrane proteins with the long, catalytic relevant C-terminal domain facing the cytosol. Topology prediction programs however, indicated that the C-tail of four AAA+ ATPases and two of three HflB of Ca. P. mali face to the outside, hence the sieve tube cytoplasm (Seemüller et al. 2013).

AAA+ proteins are of crucial importance for bacterial virulence. The ClpC ATPase from Staphylococcus aureus for example regulates transcription, which is a major virulence factor (Luong et al. 2011). Similarly, AAA+ proteins are essential for the function of secretion systems. Several bacterial species such as Salmonella enterica, Agrobacterium tumefaciens and Pseudomonas syringae use type three secretion system (TTSS) to inject virulence factors into the eukaryotic host (Van Melderen and Aertsen 2009; Alix and Blanc-Potard 2008). This unusual finding on C-tail orientation is a new aspect in understanding phytoplasma pathogenicity at the sieve tube level. It is conceivable that the powerful AAA+ proteins attack structures or components of sieve elements, in particular if the pathogens are attached to the membrane. Adherence to host membranes is well established for most mycoplasmas pathogenic to humans and animals and is considered to be a prerequisite for colonization and infection evidenced by the harm caused to the membranes (Razin at et al. 1998). However, such an attachment, which is based on protein/protein interaction, is not clearly established for phytoplasmas.

**Conclusion**

The effect of phytoplasma infections on function of the phloem and the resulting disease received little attention in the past. However, recent results in phytoplasma and phloem research, like the correlation of phytoplasma-AAA+ proteins with virulence or changes in phytohormone levels under stress situations offer the opportunity to make significant progress in understanding induction and development of phytoplasmal diseases. For the future it will be important to study the effect of the sieve tube-born inhabiting pathogens on the phloem. We suppose that after infection by different phytoplasma strains and the release of proposed effectors/proteases from plasma-membrane proteins (e.g. AAA+ proteins), the first reaction is a Ca²⁺ increase in sieve elements - resulting in filament formation of phloem-proteins and, later on, callose deposition at sieve plates and sieve pores. A subsequent reaction is the change of phytohormone dispersion, integrity of plasma membrane and change of mass flow direction (Figure 7).

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Figure 7: A proposed model about the (sub-)cellular level of plant-phytoplasma interaction.

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